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## (54) METHOD FOR *IN VITRO* PROPAGATION AND MAINTENANCE OF CELLS

(71) We, MONSANTO COMPANY, a corporation organised under the laws of the State of Delaware, United States of America, of 800 North Lindbergh Boulevard, St. Louis, Missouri 63166, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a method for propagating and/or maintaining cells in vitro. More specifically this invention relates to a method for propagating cells on a surface of a hollow fiber membrane.

The cultivation of vertibrate animal cells in vitro i.e. apart from the host animal has long been known. It is generally considered that the first such cultivation was performed in the first decade of this century and involved the growth of infectious canine lymphosarcoma in blood.

In spite of long experience this art or science has come into prominence only in recent years. This prominence is mainly due to the demand for many types of vertebrate animal cells for use in medical and veterinary research and diagnosis, in culturing of infectious agents such as viruses, and in the production of hormones and other biological products. Presently this demand is especially high for mammalian cells, particularly normal mammalian cells which must be attached to a surface for growth, as opposed to growth in a suspension culture.

Numerous procedures have been developed for propagating and/or maintaining attached cells in vitro. Perhaps the most successful prior method involves attaching and growing cells on the interior surface of glass and plastic roller tubes and bottles. Another successful method is by attaching and growing cells on the flat side of appropriately shaped stationary bottles. Many types of cells have been grown by these and other prior art methods with such methods being most successful in growing abnormal or altered cells, that is, cells which possess an abnormal or different number of chromosomes from normal cells of the same type and which have the ability to regenerate an indefinite number of times. However these and other prior methods possess several serious drawbacks, especially in the economical production of large quantities of normal or unaltered mammalian cells. Normal cells in contrast to abnormal cells possess the normal number of chromosomes for the species and regenerate only a relatively predictable number of times before senescence or death.

The principal drawback of prior methods in the propagation of normal mammalian cells arises from the fact that with such methods it is difficult to provide aerobic conditions. Stated otherwise unless the oxygen supply is properly provided in adequate quantities to normal cells the cells will not maintain their normal, differentiated functional state. An additional drawback of prior methods in propagating attached cells, whether normal or abnormal, is the difficulty encountered in attaining tissue-like densities on the growing surface because of problems pertaining to nutrient diffusion within the tissues. Further prior art methods are not readily adapted to large scale operations and thus are not economically suited for producing large quantities of cells.

It has been discovered in accordance with the present invention that cells are propagated by aseptically attaching cells to one wall of an oxygen permeable hollow fiber membrane and contacting the opposite wall of the hollow fiber membrane with an oxygen carrier thereby to cause oxygen to permeate through the membrane and to bring it into contact with the attached cells under aerobic conditions, while simultaneously incubating the attached cells in a nutrient cell culture medium.

The invention accordingly comprises a method for propagating or maintaining cells in vitro which comprises (a) contacting a suspension of cells in a cell culture medium with one wall of a non-toxic, oxygen-permeable, hollow, fiber membrane thereby to attach cells to the said wall and (b) contacting the opposite wall of the membrane with an oxygen carrier thereby to cause passage of oxygen through

the membrane and bring the oxygen into contact with the attached cells on the other side of the membrane and simultaneously incubating the cells in a nutrient cell culture medium under cell growth or maintenance conditions of pH and temperature, with oxygen being supplied in sufficient concentration to exceed the maximum demand therefor and to provide aerobic conditions for growth or maintenance of cells.

By continuously passing oxygen through the membrane from the side opposite that on which cells are attached the method of the present invention permits a continuous and, if desired, uniform supply of oxygen to reach and nourish the cells thereby facilitating aerobic propagation of the cells in desired tissue densities. The oxygen for aerobic growth is suitably supplied by utilizing a gaseous carrier containing oxygen in sufficient amount to satisfy the demand therefor.

In carrying out the method of the present invention the cells are aseptically attached to one wall or surface (exterior or interior) of the hollow fiber membrane by contacting cells suspended in a cell culture medium with the desired membrane wall. For the purpose of attachment the "cell culture medium" will typically be a nutrient medium for the cells, however a non-nutrient physiologically compatible medium such as physiological saline can also be employed if desired. Upon attachment (and during attachment if desired) oxygen is supplied to the cells by contacting the opposite side of the membrane with an oxygen carrier. Simultaneously the cells are incubated in a nutrient cell culture medium.

In a preferred embodiment the cells are attached to and grown on the exterior wall of a hollow fiber which is preferably open at both ends. By this procedure it is possible to continuously pass a stream of an oxygen carrier through the hollow core of the fiber. The continuous passage of oxygen carrier through the core of a continuously hollow fiber may be accomplished by passing the oxygen carrier through the fiber in uniform amounts or by pulsating the oxygen carrier through the fiber. Pulsation is preferred in order to obtain optimum distribution of oxygen to all cells and to minimize channeling of oxygen.

In an alternate procedure the continuously hollow fiber may be closed at one end. In this procedure the oxygen carrier is passed into the core of the fiber and oxygen is diffused through the wall and thus brought into contact with the cells. A procedure and apparatus for carrying out the method is illustrated in the flow diagram of the single Figure of the accompanying Drawing. In the figure, a reactor 1 comprises a container 2 containing a large number of hollow fibers 3 longitudinally placed in said container, with upper ends 4 projecting into a chamber 5 formed above gasket 6, and lower ends 7 projecting into chamber 8 below gasket 9. The nutrient medium is pumped from reservoir 10 through pump 11 to the reactor at inlet 12 into container 2 externally of the fibers, and can be removed from the container through outlet 13 and pump 14 to reservoir 15. Pump 14 can be operated at a flow rate slower than that of pump 11, so that less medium is removed through outlet 13 than enters through inlet 12, causing the excess to penetrate through the fiber wall and flow through the hollow fibers. The reactor is provided with oxygen by pumping air from cylinder 16, containing air and 3% carbon dioxide, through pump 17 and conduit 18 to chamber 5 of container 2, so that the air enters the open ends 4 of the hollow fibers. A pulser 19 is connected to conduit 18. The pulsesr 19, comprises a chamber 20 containing a diaphragm 21 separating upper and lower portions of said chamber and preventing movement of air therebetween. The upper part of the pulser is connected to a valve 22 which can be positioned to provide access to a vacuum, or to a source of air pressure, and which is controlled by a solenoid electrically connected to a timer 23. When valve 22 is open to the air source, the diaphragm 19 is distended downward to substantially fill the lower part of chamber 20 to provide a surge of air entering chamber 5 and flowing through the hollow fibers. The chamber 20 and diaphragm 19 are preferably sized to have sufficient volume in the surge to slightly exceed the total internal volume of the hollow fibers in the reactors, so that the surge of air can substantially remove all the materials from the cores of the fibers. The diaphragm can be made of rubber or other suitable material. The air and other material leaves the fibers through their lower ends 7 and chamber 8 through conduit 24 to overflow vessel 25. Since liquid may have penetrated the fiber, by diffusion, or because more liquid was pumped into the reactor than removed therefrom through outlet 13, liquid is generally removed and conveyed through conduit 24 to overflow vessel 25. Conduit 24 is also connected through valve 26 to cylinder 16, but this is only for the purpose of using the cylinder as an oxygen source for calibration purposes, and valve 26 is normally closed. The overflow vessel 25 is connected to a reservoir 27, and is provided with instruments 28 and 29 to provide pH and oxygen measurements at instruments 30 and 31. The reactor is provided with means for temperature control by controller 32. Refrigerator 33 provides refrigeration of components 10, 15, and 27.

The described apparatus can, for example, be operated with a 5 ml/minute air flow rate, and a liquid medium input of 0.4 ml/minute and output through outlet 13 of 0.1 ml/minute. This leaves 0.3 ml/minute of liquid medium to penetrate the fibers and to exit

Attachment of cells to an otherwise suitable membrane may be promoted by coating the surface to which the cells are to be attached with collagen.

The optimum dimensions for the hollow fibers may vary depending among other things on the apparatus and the oxygen carrier employed. Generally the inside diameter of the hollow fiber is in the range of from about 10 to about 300 microns with an inside diameter of 50—100 microns being preferred. The membrane wall must of course be sufficiently thin to permit permeation as desired and sufficiently thick so as to not rupture under the conditions employed. Typically suitable membranes have an effective wall thickness of from about 10 to about 100 microns.

Suitable cells for propagation in accordance with the method of the present invention include tissue cells from vertebrate animals which are capable of attachment and growth or maintenance on a surface. Of course cells which are inherently incapable of proliferation such as erythrocytes cannot be employed in the method of this invention. Examples of such suitable cells include diploid cell lines such as W1-38 human lung fibroblasts, MRC-5 male human fetal lung fibroblasts and DBS-FRh L-2 rhesus monkey fetal lung fibroblasts; primary cells such as bovine and human anterior pituitary cells, chicken embryo, frog epithelium and rat liver; and established cell lines such as Hela human cervix (carcinoma) cells, rhesus monkey kidney cells (LLC-$MK_2$) or Syrian baby hampster kidney cells (BHK-21).

It will be appreciated that the above list of cells is given for illustrative purposes and that other cells from other sources including avian, mammalian, reptilian and amphibian sources including normal and abnormal cells can be propagated and maintained in accordance with the method of the present invention.

The following examples illustrate specific embodiments of the invention. In the examples the preparation of the cells, the preparation of the innoculum, and the cell culturing experiments were carried out under sterile conditions.

## EXAMPLE 1

Preparation of Cells

One calf pituitary obtained by dissection from a freshly slaughtered animal was stored approximately four hours in phosphate buffered salts (PBS) medium of the following composition:

| | |
|---|---|
| NaCl | 8.0 grams |
| KCl | 0.2 grams |
| $Na_2HPO_4$ | 1.15 grams |
| $CaCl_2$ | 0.1 grams |
| $KH_2PO_4$ | 0.2 grams |
| $MgCl_2 . 6H_2O$ | 0.1 grams |
| Penicillin | * 100,000 |
| Streptomycin | 0.1 grams |
| Distilled Water | 900 mls. |

* I.U.

The temperature of the medium during storage was about 25°C. The anterior portion was dissected from the gland, cleaned to remove connective tissue and minced. The minced anterior portion was gently mixed with an aqueous solution of trypsin in a Petri dish and the resulting mixture was allowed to stand under sterile conditions for 18 hours at room temperature to obtain release of individual cells into the fluid. The aqueous trypsin solution was prepared by mixing 10 milliliters of PBS with 250,000 units of dry powdered trypsin enzyme sold under the name Tryptar by Armour and Co., Chicago, Illinois and 0.75 ml. of 0.5 normal sodium hydroxide. The released pituitary cells (epithelia) were separated from remaining connective tissue by repeated centrifugation, filtration and washing. It was determined from a cell count (with a hemocytometer) that the resulting washed cell suspension contained $1.37 \times 16^6$ cells per ml.

Preparation of Innoculum

To prepare the innoculum 30 mls. of the washed cell suspension were diluted to 150 mls. with Basal Medium Eagle's (BME) containing 10% fetal calf serum. The composition of the Basal Medium Eagle's (BME) constituting 90% of the $BME_{90}$ fetal $calf_{10}$ was as follows:

| | Mg/l. |
|---|---|
| 1-arginine chlorhydrate | 105 |
| 1-cystine | 24 |
| 1-histidine monohydrochlorhydrate | 31 |
| 1-isoleucine | 52 |
| 1-lysine chlorhydrate | 58 |
| 1-leucine | 52 |
| 1-methionine | 15 |
| 1-phenylalanine | 32 |
| 1-threonine | 48 |
| 1-tryptophan | 10 |
| 1-tyrosine | 36 |
| 1-valine | 46 |
| choline chloride | 1.00 |
| Folic acid | 1.00 |
| Isoinositol | 1.00 |
| Nicotinamide | 1.00 |
| Pantothenic acid | 1.00 |
| Pyridoxal | 1.00 |
| Thiamine | 1.00 |
| Riboflavin | 0.10 |
| NaCl | 6800 |
| KCl | 400 |
| $NaH_2PO_4 . 2H_2O$ | 150 |
| $NaHCO_3$ | 2000 |
| $CaCl_2$ | 200 |
| $MgCl_2$ | 200 |
| Glucose | 1000 |

with 5 ml/minute of gases. In addition to the gaskets 6 and 9, the fibers are sealed to each other near their ends by a resinous compound to prevent movement of material from the interstices of the fibers into chambers 5 and 8.

The parts of the apparatus can, of course, be varied in size. However, a reactor of overall length 25 cm. with fiber length of about 20 cm. can be employed, with about 15 cm. of the fiber available for cell growth. Such a reactor may have about 5 cm. overall diameter, with container 2 having an outer diameter of 2.38 cm. and inner diameter of 1.9 cm. The container can contain about 1000 fibers of 360 micrometer outer diameter and 200 micrometer inner diameter, for a total effective hollow core volume of about 6.3 ml. The pulser 19 has an internal volume of about 28 ml. and the diaphragm can displace about 8 ml. of the lower portion of the chamber 20, so that the surge is adequate to sweep out the fibers. The surge gives a pressure differential of 3 to 5 psi. The pulse can be operated at various frequencies, for example a cycle of 10 seconds on vacuum and 10 seconds on air pressure, with repetition of the cycle. If desired, the cycle can be varied by using a different duration for the air pressure and vacuum phases of the pulser, and the pulser can also be operated to provide irregular surges of air, rather than a regular cycle. Also, the surge may have varying forms, for example a short sharp rise in pressure, followed by a slow fall, or a regular moderate rise and moderate fall, etc., sine wave characteristics, etc. It is not necessary to have the flow fall to zero between surges, although this provides acceptable results, and in fact, the flow can even be reversed, if desired, at some stages of the pulsation. The reactor can be operated at varying rates, etc., but illustrative rates are, for example, 5 ml/minute air flow and .4 ml/minute liquid input, with 0.3 ml of the liquid penetrating the fibers and exiting from the ends thereof.

In the method of the present invention any suitable oxygen carrier may be employed. Generally air is the preferred oxygen carrier; however, carriers containing dissolved oxygen such as silicone polymers, hemoglobin, fluorocarbons, and oxygenated nutrient medium may also be used with desired results, although special procedures or conditions may then be necessary to satisfy the oxygen demand to obtain aerobic growth. When air or other suitable gaseous mixtures or nitrogen and oxygen are employed it is also preferred that the gas contain small amounts of carbon dioxide e.g. on the order of 2—5%. The carbon dioxide serves to provide carbonate buffering and thereby assists in maintaining the pH of the medium on the other side of the membrane within the desired range.

The cells are incubated in a nutrient cell culture medium under cell growth maintenance conditions of pH and temperature. Suitable nutrient cell culture media are known to the art and such may be used in the method of the present invention. Typically such nutrient culture media contain the known essential amino acids, vitamins, carbohydrates, mineral salts and, preferably, blood serum. Fungicides and bacteriacides may also be included in such media in desired amounts to prevent the growth of undesired microorganisms. As indicated above the pH of the nutrient medium is advantageously controlled within the desired range (typically in the range of 6.8—8.2) by including small amounts of carbon dioxide in the oxygen carrier. However if desired the pH can be controlled by including a suitable buffer such as HEPES buffer (a mixture of N - 2 - hydroxyethyl piperazine and N' - 2 - ethane sulfonic acid) in the nutrient cell culture medium itself. Other suitable methods for controlling pH such as passing the medium over ionic exchange resins may also be employed.

The choice of temperature for incubation of cells is within the skill of the worker in the field of cell and tissue culturing and will depend principally upon the physiological temperature for the particular cells to be propagated, that is the optimum temperature at which growth or maintenance of the cells occurs. For example when normal mammalian cells are propagated a narrow temperature range of from about 35—40°C is typically employed whereas, for example, if the cells are reptilian in origin lower or higher temperatures may be employed.

The method of the present invention utilizes hollow fiber membranes. The hollow fiber membranes may be employed in any suitable fashion such as for example in bundles, in single strands or in mesh relationship. The hollow fiber is, of course, designed to be permeable to gas, but impermeable to the cells. The membrane can be of a dense or of a "Leob" structure. (A Loeb membrane is an anisotropic membrane having a thin layer or "skin" on top of a thicker layer having an open cellular structure that has little resistance to transfer of water or other solvents.) The hollow fiber may be produced from any suitable material which is non-toxic to the cells which can be appropriately spun into fibers and which permits cell attachment thereto. Examples of such materials include polyolefins such as polyacrylonitrile and polystyrene, polyionic polymers, polycarbohydrates such as cellulose, and cellulose derivatives, for example, cellulose esters, polypeptides such as collagen, silicone rubber polymers or fluorocarbon polymers. It has been found that cell attachment to the surface of the membrane is promoted when the membrane possesses increased surface energy as is evidenced by the presence of positive or negative charges.

| | Mg/l |
|---|---|
| 1-glutamine | 212 |
| Phenol red | 20 |
| Penicillin | (1) |
| Streptomycin | 50 |

(1) 50,000 I.U.

Reactor

The culturing of cells was carried out using a cell culture reactor consisting of a bundle of 100 open ended continuously hollow polymeric fibers, in a U position in a 10 ml. glass flask. The two ends of the fiber bundles are fitted in separate holes of a three-holed rubber stopper with the stopper being positioned in the neck of the flask. The third hole of the stopper is available for introduction and extraction of medium. The hollow fiber material is a commercial polyionic polymer material based on a copolymer of 40% by weight acrylonitrile and 60% by weight vinyl chloride and sold under the name Amicon X M-50 by Amicon Corp., Lexington, Massachusetts, U.S.A. Each fiber is 10 cms. in length and has approximately 1/2 sq.cm. of cell growing surface. Each fiber has an internal diameter of 360 microns and a wall thickness of 80 microns with the wall having a "Loeb" configuration.

Cell Culturing

The reactor was sterilized using beta-propiolactone vapors and then rinsed with phosphate buffer. Innoculum (8 milliliters) prepared above was introduced into the reactor to attach cells to the outer walls of the fibers. The reactor was placed in a jacketed carbon, dioxide incubator and the contents were incubated for 49 days at 37°C. Throughout the 49 days incubation period a filtered mixture of air with 3% carbon dioxide was pumped (with an air pump) through the interior of the hollow fibers. During incubation the medium was changed at two day intervals. The withdrawn medium was collected and retained for analysis. On completion of the incubation period the medium was withdrawn from the reactor and a heavy confluent growth of cells on the hollow fibers was observed. The cells were then prepared for microscopic examination by formaldehyde fixation and staining on the fibers. It was observed by microscopic examination that the cells were normal. The retained media was analyzed for lactic acid and growth hormone. The analysis showed that the total retained media contained 700 nanograms of growth hormone and that the media were substantially free of lactic acid. The substantial absence of lactic acid indicates that the cell growth was achieved under aerobic conditions.

As a control 10 mls. of the above-prepared cell innoculum medium was placed in each of two "T" flasks and the medium was incubated in the jacketed carbon dioxide reactor at 37°C for 49 days (simultaneously with the cell culture reactor). Medium was changed at two day intervals and the withdrawn medium was collected and retained. The area of growth for each "T" flask was 50 sq.cm. The retained medium was analyzed for growth hormone and lactic acid. The analysis showed that the combined media from both flasks contained 800 nanograms of growth hormone, (an average of 400 nanograms per flask) and that the production of lactic acid (based on glucose) was quantitative. The quantitative production of lactic acid indicates anaerobic cell growth condition.

## EXAMPLE 2

In this example the cell culture reactor employed is of the type described in the United States patent specification No. 3,228,877. The reactor consists of a bundle of 1000 continuously hollow fibers (Amicon XM-50) having a total external surface area of about 900 sq.cms. contained in a tubular casing. The hollow fibers are open at each end to permit continuous flow of the oxygen carrier. A cell innoculum medium of porcine pituitary cells ($7.6 \times 10^5$ cells per ml) was prepared following the procedure of Example 1. The cell culture reactor was innoculated with 33 mls. of innoculum medium to attach cells to the outer surface of the fibers. Incubation at 37°C was conducted for six days to produce a confluent growth of cells under aerobic conditions on the fibers. During the six day period air with 3% carbon dioxide was passed through the fibers in a pulsating manner (1 pulse every 10 seconds) at a rate of 5 cc. per minute. Throughout the six day period the cell culture medium was continuously changed. After initial introduction of the innoculum the medium change was accomplished by continuously pumping medium ($BME_{99}$Fetal Calf $_1$) into the reactor in contact with the exterior walls of the fibers at the rate of 4 mls. per minute and out of the reactor (excluding medium discharged through the fibers) at the rate of 1 ml. per minute. By this procedure medium flows radially through the cell layers, through the walls of the fibers and into the hollow cores of the fibers. Discharged medium was analyzed and found to contain growth hormone.

Radial flow of medium as in the procedure of this Example provides for the optimum distribution of nutrients to the cells thus aiding in the formation of dense cell layers. Since with radial flow the smaller molecules more readily permeate the hollow fiber membrane than do the larger molecules, the procedure further serves to concentrate large molecules (fetal calf serum, hormones and other metabolites) on the other side of the membrane.

## EXAMPLE 3

The general procedure of Example 2 was repeated with the exception that the oxygen carrier was oxygenated Basal Medium Eagle's without serum which was pumped through the interior of the hollow fibers at the rate of 12 mls. per minute. The incubation was carried out for 12 days. Heavy confluent cell growth was observed on the fibers. Cell growth was aerobic during the first four days and anaerobic thereafter.

## EXAMPLE 4

Human embryonic lung fibroblasts (WI-38 cells at 26th generation isolated by L. Hayflick) were incubated following the general procedure of Example 2 for 24 days at 37°C in a cell culture reactor consisting of a bundle of polymeric hollow fibers (Amicon XM-50 polymer) in a rectangular casing. The fibers are open at each end to permit continuous flow of oxygen carrier. The pH of the cell culture medium throughout the 24 day incubation period fluctuated in the range of from about 7.2 to about 7.9. The hollow fiber bundle had a total external surface area of about 85 $cm^2$ of which about 65 $cm^2$ of the surface area was continuously immersed in medium throughout the 24 day incubation period. The cells were attached to the external walls of the fibers as in Example 2 with approximately 58% of the cells being attached to the external walls of the fibers after 18 hours. The cell density on the fibers after the 18 hour period was $1.56 \times 10^4/cm^2$ of surface. On completion of the 18 hour attachment period medium was pumped into and through the reactor in contact with the exterior walls of the fibers at the rate of 4 mls/hour and oxygen carrier (air+3% $CO_2$) was passed through the fibers at the rate of 40 mls/hour. To obtain optimum contact of medium with the cells the fibers in the bundle were maintained in a spread position and the medium was pumped into the reactor perpendicular to the fibers. Confluent growth of cells on the fibers was obtained after 10 days of incubation with a cell density of $1—1.5 \times 10^5$ $cm^{-2}$ being observed. After the 14th day of incubation the cell density was $7.5 \times 10^5$ cells/$cm^2$. The cells were maintained for an additional 10 days with no increase in cell density being observed after the 14th day of incubation. After the 24 day incubation period the cells were removed from the fibers by trypsinization. The cells gave a normal appearance upon microscopic examination.

It is greatly advantageous to operate the present process using a gaseous oxygen system to ensure adequate oxygen for cell maintenance or growth under aerobic conditions. By use of air or other gases in the fiber, it is possible to supply oxygen at a much greater rate than when using a solution of oxygen in a liquid. Air has 0.0029 grams oxygen per ml., compared to much lower quantities of oxygen that are soluble in most liquids. Also the rate of diffusion is rapid in gases but much slower in liquids. Air is about 4000 times as effective in providing oxygen as is an aqueous system saturated with oxygen. Other gaseous systems containing oxygen are suitably used, and any system with a partial gas phase, such as a foam, is included herein as a gaseous system. The amount of oxygen needed in the oxygen carrier will vary with the flow rate and other factors, but oxygen concentrations greater than 50 to 100 micrograms per ml. are generally suitable. The practical flow rates are limited on the upper side by the strength of the fibers, and use of high oxygen concentrations lessens the need for high flow rates. A gaseous system can employ various other gases, as a diluent, along with oxygen. Generally it will be desirable that such diluent be inert to the cell culture, or at least not known to have any strong adverse effect on cell cultures, and that such diluents not react readily with oxygen to use up the available oxygen. The present process can use atmospheric, sub-atmospheric, or super-atmospheric pressures and if, for some reason, it is desired to operate at sub-atmospheric pressures, oxygen can be utilized at about 0.2 atmosphere, and it is then unnecessary to use any diluent for the oxygen. The present process will not generally employ oxygen in such high concentrations that cell growth or maintenance conditions are not provided, since too high a concentration can cause the death of significant numbers of cells. In the production of cells, it is desirable to achieve high production and growth rates. Thus in the present process it is desirable to supply enough oxygen in excess of the maximum demand therefor to provide aerobic growth or maintenance at the maximum cell production rate and with the maximum cell density ultimately achieved, and to do so under long term culturing. Of course, some of the benefits of the process are achieved if the cell growth occurs under aerobic conditions with more than adequate oxygen for a time, even if ultimately some anaerobic growth occurs because of the production of many layers of cells which impede oxygen diffusion. Thus, the present invention includes a method where adequate oxygen is provided for aerobic growth at maximum or high cell density, even though such growth may not be achieved after a period of time under some conditions.

## WHAT WE CLAIM IS:—

1. A method for propagating or maintaining cells in vitro which comprises (a) contacting a suspension of cells in a cell culture medium with one wall of a non-toxic, oxygen-permeable, hollow, fiber membrane thereby to attach cells to the said wall and (b) contacting the opposite wall of the membrane

with an oxygen carrier thereby to cause passage of oxygen through the membrane and bring the oxygen into contact with the attached cells on the other side of the membrane and simultaneously incubating the cells in a nutrient cell culture medium under cell growth or maintenance conditions of pH and temperature, with oxygen being supplied in sufficient concentration to exceed the maximum demand therefor and to provide aerobic conditions for growth or maintenance of cells.

2. The method of Claim 1, wherein the suspension is contacted with the exterior wall of the fibre membrane having open ends that are inaccessible to the suspension, and the oxygen carrier is passed through the interior of the hollow fiber membrane.

3. The method of either of Claims 1 and 2, in which oxygen is supplied in a gaseous system.

4. The method of any of the preceding Claims, wherein the cells are normal mammalian cells.

5. The method of Claim 4, wherein the normal mammalian cells are human cells.

6. The method of either of Claims 4 and 5, wherein the normal mammalian cells are pituitary cells.

7. The method of any of the preceding Claims, wherein the oxygen carrier comprises air.

8. The method of any of the preceding Claims, in which the flow of the oxygen carrier is characterized by intermittent surges.

9. The method of Claim 2 or of any of Claims 3 to 8 as dependent on Claim 2, wherein nutrient medium is provided externally to the fiber in a manner so that medium flows through the wall of the fiber and into the interior thereof.

10. The method of any of the preceding Claims, in which air with pulsated flow is utilized as the oxygen carrier.

11. A method according to Claim 1, of propagating or maintaining cells, substantially as described in any of the Examples herein.

12. Cells that have been propagating or maintained in vitro by a method according to any of the preceding Claims.

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